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(54) Titre : UTILISATION DE GAL α (1,3)GALACTOSYLE TRANSFERASE DANS LES THERAPIES PAR GREFFE
ENTRE DEUX ESPECES

(54) Title: USE OF PORCINE GAL α (1,3) GALACTOSYL TRANSFERASE IN XENOGRAFT THERAPIES

(57) Abrégé/Abstract:

DNA sequences encoding a porcine Gal α (1,3) galactosyl transferase and clones containing such sequences are provided. The porcine Gal α (1,3) galactosyl transferase produces the Gal α (1,3) Gal epitope on the surfaces of porcine cells. This epitope is recognized by human anti-Gal α (1,3) Gal antibodies which are responsible for hyperacute rejection of xenotransplanted pig cells, tissues and organs. Methods of reducing such hyperacute rejection are also provided.



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| <p>(54) Title: USE OF PORCINE GAL α(1,3) GALACTOSYL TRANSFERASE IN XENOGRAFT THERAPIES</p> <p>(57) Abstract</p> <p>DNA sequences encoding a porcine Galα(1,3) galactosyl transferase and clones containing such sequences are provided. The porcine Galα(1,3) galactosyl transferase produces the Galα(1,3) Gal epitope on the surfaces of porcine cells. This epitope is recognized by human anti-Galα(1,3) Gal antibodies which are responsible for hyperacute rejection of xenotransplanted pig cells, tissues and organs. Methods of reducing such hyperacute rejection are also provided.</p> | | |

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WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising:
 - (a) the nucleic acid sequence of SEQ ID NO: 1; or
 - (b) an antisense sequence complementary to (a); or
 - (c) both (a) and (b).
2. An isolated nucleic acid molecule comprising:
 - (a) the nucleic acid sequence of SEQ ID NO:2; or
 - (b) an antisense sequence complementary to (a); or
 - (c) both (a) and (b).
3. Clone pPGT-4 having deposit designation number AGAL N94/9030.
4. Clone pPGT-2 having deposit designation number AGAL N94/9029.
5. Clone λ PGT-g1 having deposit designation number AGAL N94/9027.
6. Clone λ PGT-g5 having deposit designation number AGAL N94/9028.
7. A porcine cell comprising an inactivated porcine $\alpha(1,3)$ galactosyl transferase gene, said inactivated porcine $\alpha(1,3)$ galactosyl transferase gene comprising a wild type porcine $\alpha(1,3)$ galactosyl transferase sequence disrupted by a cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence wherein the cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence comprises a mutation of SEQ ID NO:1 wherein the mutation is selected from the

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group consisting of a deletion, an insertion, a substitution, and an addition such that the cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence does not encode a functional galactosyl transferase so that immune reaction of the cell with human antibodies reactive with $\text{Gal}\alpha(1,3)\text{Gal}$ epitopes is avoided.

8. A porcine cell comprising an inactive porcine $\alpha(1,3)$ galactosyl transferase gene, said inactivated porcine $\alpha(1,3)$ galactosyl transferase gene comprising a wild type porcine $\alpha(1,3)$ galactosyl transferase sequence disrupted by a cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence wherein the cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence comprises a mutation of SEQ ID NO:2, wherein the mutation is selected from the group consisting of a deletion, an insertion, a substitution, and an addition such that the cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence does not encode a functional galactosyl transferase so that immune reaction of the cell with human antibodies reactive with $\text{Gal}\alpha(1,3)\text{Gal}$ epitopes is avoided.

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5 Use of Porcine Gal α (1,3) galactosyl transferase in xenograft therapies

 This invention relates to xenotransplantation
(transplantation across species) and is particularly
concerned with methods of alleviating xenotransplant
10 rejection, maintenance of xenotransplanted tissue in an
animal, nucleotide sequences useful in xenotransplant
therapies, rejection resistant transgenic organs, and
transgenic animals whose tissues are rejection-resistant
on xenotransplantation.

15 The current shortage of tissues for human
transplantation has led to recent close examination of
xenografts as a possible source of organs. However, when
tissues from non human-species are grafted to humans,
hyperacute rejection occurs due to the existence of
20 natural antibodies in human serum which react with
antigens present in these species, with rejection
occurring within 10-15 minutes of transplantation. This
phenomenon depends, in general, on the presence of some
or all of antibody, complement, neutrophils, platelets
25 and other mediators of inflammation. In transplantation
of vascularized organs between "discordant" species
(those in which natural antibodies occur) the first cells
to encounter natural antibodies are the endothelial cells

lining the blood vessels and it is likely that activation of these cells is induced by antibody binding to xenoantigens or other factors, leading to hyperacute rejection.

There is considerable uncertainty in the art concerning the nature of possible target xenoantigens on xenograft tissues. Platt et al (Transplantation 50:817-822,1990) and Yang et al (Transplant. Proc. 24:593-594, 1992) have identified a triad of glycoproteins of varying molecular weights as the major targets on the surface of pig endothelial cells. Other investigators (Holgersson et al, Transplant Proc 24:605-608, 1992) indicate glycolipids as key xenoantigens.

We have now found that xenograft rejection, particularly in the context of pig tissue, is associated with antibodies reactive with galactose in an $\alpha(1,3)$ linkage with galactose, (the $\text{Gal}\alpha(1,3)\text{Gal}$ epitope) modulating the interaction between antibodies reactive with the $\text{Gal}\alpha(1,3)\text{Gal}$ epitope of xenotransplant tissue effects rejection.

According to one aspect of the invention, there is provided an isolated nucleic acid molecule comprising:

- (a) the nucleic acid sequence of SEQ ID NO: 1; or
- (b) an antisense sequence complementary to (a); or
- (c) both (a) and (b).

According to another aspect of the invention, there is provided an isolated nucleic acid molecule comprising:

- (a) the nucleic acid sequence of SEQ ID NO:2; or
- (b) an antisense sequence complementary to (a); or
- (c) both (a) and (b).

According to a further aspect of the invention, there is provided a clone pPGT-4 having deposit

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designation number AGAL N94/9030.

According to another aspect of the invention, there is provided a clone pPGT-2 having deposit designation
5 number AGAL N94/9029.

According to a further aspect of the invention, there is provided a clone λ PGT-g1 having deposit designation number AGAL N94/9027.

According to another aspect of the invention, there
10 is provided a clone λ PGT-g5 having deposit designation number AGAL N94/9028.

According to a further aspect of the invention, there is provided a porcine cell comprising an inactivated porcine $\alpha(1,3)$ galactosyl transferase gene,
15 the inactivated porcine $\alpha(1,3)$ galactosyl transferase gene comprising a wild type porcine $\alpha(1,3)$ galactosyl transferase sequence disrupted by a cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence wherein the cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence
20 comprises a mutation of SEQ ID NO:1 wherein the mutation is selected from the group consisting of a deletion, an insertion, a substitution, and an addition such that the cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence does not encode a functional galactosyl
25 transferase so that immune reaction of the cell with human antibodies reactive with Gal $\alpha(1,3)$ Gal epitopes is avoided.

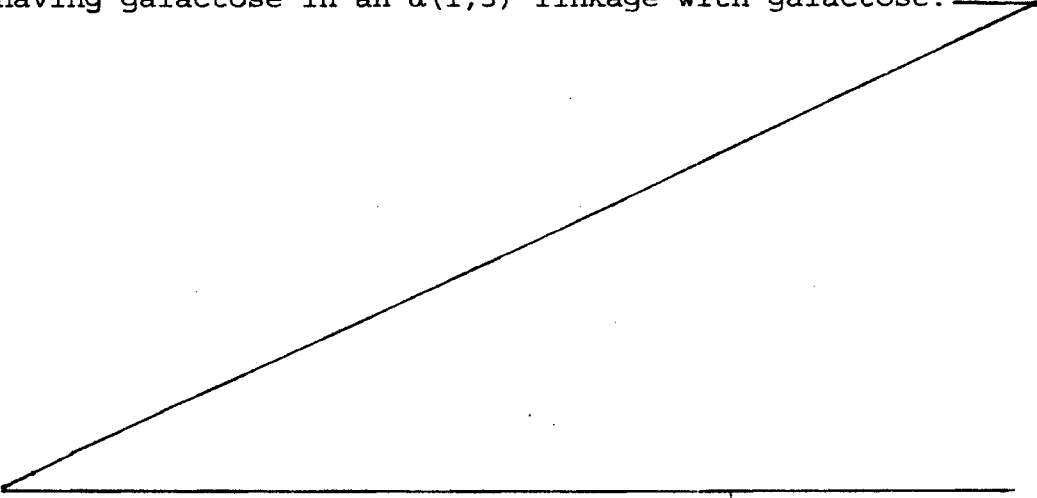
According to another aspect of the invention, there is provided a porcine cell comprising an inactive porcine
30 $\alpha(1,3)$ galactosyl transferase gene, the inactivated porcine $\alpha(1,3)$ galactosyl transferase gene comprising a wild type porcine $\alpha(1,3)$ galactosyl transferase sequence

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disrupted by a cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence wherein the cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence comprises a mutation of SEQ ID NO:2, wherein the mutation is selected from the group consisting of a deletion, an insertion, a substitution, and an addition such that the cloned mutant porcine $\alpha(1,3)$ galactosyl transferase sequence does not encode a functional galactosyl transferase so that immune reaction of the cell with human antibodies reactive with Gal $\alpha(1,3)$ Gal epitopes is avoided.

According to a further aspect of the invention, there is provided a method for blocking human anti-Gal $\alpha(1,3)$ Gal antibodies comprising changing the conformation of the antibody reactive site so as to reduce the affinity of the antibody for the Gal $\alpha(1,3)$ Gal epitopes.

In accordance with the first aspect of this invention, there is provided a method of inhibiting xenotransplant rejection in an animal patient, comprising administering to the patient an effective amount of an antagonist of antibody binding to xenotransplant antigens having galactose in an $\alpha(1,3)$ linkage with galactose.



Another aspect of this invention relates to the maintenance of xenograft tissue in an animal, which comprises administering to the animal a graft rejection effective amount of an antagonist to antibodies which
5 bind to the xenograft antigen epitope Gal α (1,3)Gal.

In another aspect of this invention there is provided a method of inhibiting the binding of antibodies to the Gal α (1,3)Gal epitope which comprises modulating the interaction between the antibodies and the epitope
10 with an antagonist which blocks the binding of the antibodies to the Gal α (1,3)Gal epitope.

Preferably the xenograft recipient is a human. Age is not a determining factor for xenograft transplantation although transplants in the elderly over 75 years would
15 normally not be carried out. The invention is directed particularly to human transplantation with xenograft tissue.

Xenografted tissue is preferably of pig origin. Tissues from other mammals are also contemplated for use
20 in this invention. Preferably the xenotransplanted tissue is in the form of an organ, for example, kidney, heart, lung or liver. Xenotransplant tissue may also be in the form of parts of organs, cell clusters, glands and the like. Examples include lenses, pancreatic islet
25 cells, skin and corneal tissue. The nature of the xenotransplanted tissue is not of itself critical as any xenotransplanted tissue which expresses antigens having

Gal α (1,3)Gal epitopes may be utilized in accordance with this invention.

The binding of antibody to the Gal α (1,3)Gal epitope expressed on xenotransplanted tissue provokes rejection of the tissue by humoral as well as cell-mediated immune effects leading to tissue rejection in a very short time scale, such as less than one hour. Antagonists which antagonize the binding of antibodies to the Gal α (1,3)Gal epitope block antibody binding and therefore inhibit xenotransplant rejection. Because antibody binding is blocked, immune responses which give rise to tissue rejection are prevented.

In accordance with a further aspect of this invention, there is provided an antagonist which modulates the interaction of antibodies directed against Gal α (1,3)Gal.

Any antagonist capable of modulating the interaction between antibodies directed to the Gal α (1,3)Gal linkage may be utilized in this invention. By reference to modulation, is meant blockage of antibody binding or decrease in affinity reactivity of antibodies for the Gal α (1,3)Gal epitope. Various mechanisms may be associated with the blockage of antibody binding or decreased affinity of antibodies for their respective epitope. These include binding or association with the antibody reactive site and change of conformation of the antibody reactive site, such as by binding to residues associated with, adjacent to, or distanced from the

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active site, which effect the conformation of the active site such that it is incapable of binding the Gal α (1,3)Gal epitope or binds the epitope with reduced affinity. For example, in accordance with techniques well known in the art (see, for example, Coligan, et al., eds. Current Protocols In Immunology, John Wiley & Sons, New York, 1992; Harlow and Lane, Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; and Liddell and Cryer, A Practical Guide To Monoclonal Antibodies, John Wiley & Sons, Chichester, West Sussex, England, 1991), such a change of the conformation of the antibody reactive site can be achieved through the use of an anti-idiotypic antibody raised against the natural antibody or fragments thereof. As is also well known in the art, these anti-idiotypic antibodies may be modified to enhance their clinical usefulness, for example by enzymatic techniques such as preparing Fab' fragments, or by recombinant techniques such as preparing chimeric, humanized, or single chain antibodies.

This invention is not limited to any specific antagonist and any antagonist which is non-toxic and which modulates the interaction between antibodies specific for the Gala(1,3)Gal epitope may be used in this invention. Suitable examples of antagonists include D-galactose and melibiose, stachyose and methyl- α -D-galactopyranoside, D-galactosamine and derivatives thereof. The term derivatives encompasses, for example, any alkyl, alkoxy, alkylkoxy, aralkyl amine,

hydroxyl, nitro, heterocycle, sulphate and/or cycloalkyl substituents whether taken alone or in combination, which derivatives have antagonist activities. This may be assessed according to methods as herein described.

5 Carbohydrate polymers containing one or more of the aforesaid carbohydrate moieties or derivatives may also be utilized in this invention.

The amount of antagonists which is effective to modulate interaction between antibodies reactive with
10 Gal α (1,3)Gal epitopes will vary depending upon a number of factors. These include the nature of the animal being treated, the nature of species of the transplanted tissue, the physical condition of the transplant recipient (age, weight, sex and health) and the like. In
15 respect of human transplant recipients of tissue, for example from pigs, the amount of antagonists administered will generally depend upon the judgement of a consulting physician. As an example, a graft rejection effective amount of an antagonist in human subjects may be in the
20 order of from 0.01mg to 1000gm per dose, more preferably 10mg to 500mg, more preferably 50mg to 300mg, and still more preferably 50mg to 200mg per dose.

The schedule of administration of antagonists to inhibit rejection and maintain xenografts will depend
25 upon varying factors as mentioned above. Varying dosage regimes may be contemplated, such as daily, weekly, monthly or the like.

The mode of administration of antagonists and dosage forms thereof are not critical to this invention. Antagonists may be administered parenterally (intravenous, intramuscular or intraorgan injection),
5 orally, transdermally, or by vaginal or anal routes, or by other routes of administration, as are well known in the art. Antagonists may be in solid or liquid form and would generally include pharmaceutically acceptable or veterinarily acceptable excipients and/or carriers.
10 Examples of dosage forms which may be used in this invention are those well known in the art as mentioned previously such as described in Remington's Pharmaceutical Sciences (Mack Publishing Company, 10th Edition.

15 In still another aspect of this invention, there is provided nucleotide sequences encoding $\alpha(1,3)$ galactosyl transferase and mutants thereof. Preferably, the nucleotide sequence encodes pig $\alpha(1,3)$ galactosyl transferase.

20 Nucleotide sequences may be in the form of DNA, RNA or mixtures thereof. Nucleotide sequences or isolated nucleic acids may be inserted into replicating DNA, RNA or DNA/RNA vectors as are well known in the art, such as plasmids, viral vectors, and the like (Sambrook et al,
25 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY, Second Edition 1989).

Nucleotide sequences encoding $\alpha(1,3)$ galactosyl transferase may include promoters, enhancers and other

regulatory sequences necessary for expression, transcription and translation. Vectors encoding such sequences may include restriction enzyme sites for the insertion of additional genes and/or selection markers,
5 as well as elements necessary for propagation and maintenance of vectors within cells.

Mutants of nucleotide sequences encoding $\alpha(1,3)$ galactosyl transferase are particularly preferred as they may be used in homologous recombination techniques as are well known in the art (Capecchi M R, Altering the Genome by Homologous Recombination, Science 244:1288-1292, 1989; Merlino G T, Transgenic Animals in Biomedical research, FASEB J 5:2996-3001, 1991; Cosgrove et al, Mice Lacking MHC Class II Molecules, Cell 66:1051-1066, 1991; Zijlstra et al, Germ-line Transmission of a disrupted B2-microglobulin gene produced by homologous recombination in embryonic stem cells, Nature 342:435, 1989) for the inactivation of wild type $\alpha(1,3)$ galactosyl transferase genes.
10
15

Mutant $\alpha(1,3)$ galactosyl transferase nucleotide sequences include nucleotide deletions, insertions, substitutions and additions to wild type $\alpha(1,3)$ galactosyl transferase such that the resultant mutant does not encode a functional galactosyl transferase.
20
25 These nucleotide sequences may be utilized in homologous recombination techniques. In such techniques, mutant sequences are recombined with wild type genomic sequences in stem cells, ova or newly fertilized cells comprising

from 1 to about 500 cells. Nucleotide sequences utilized in homologous recombination may be in the form of isolated nucleic acids sequences or in the context of vectors. Recombination is a random event and on
5 recombination, destruction of the functional gene takes place.

Transgenic animals produced by homologous recombination and other such techniques to destroy wild type gene function are included within this invention, as
10 are organs derived therefrom. By way of example, transgenic pigs may be produced utilizing homologous recombination techniques to produce a transgenic animal having non-functional $\alpha(1-3)$ galactosyl transferase genomic sequences. Tissues derived from such transgenic
15 animals may then be utilized in xenotransplantation into human patients with the avoidance of immune reaction between circulating human antibodies reactive with $\text{Gal}\alpha(1-3)\text{Gal}$ epitopes. Such transplants are contemplated to be well tolerated by transplant recipients. Whilst
20 transplanted tissue may comprise other antigens which provoke immune reaction beyond those associated with $\text{Gal}\alpha(1-3)\text{Gal}$ epitopes, removing the major source of the immune reaction with such transplanted tissues should lead to xenotransplants being relatively well tolerated
25 in conjunction with standard rejection therapy (treatment with immune suppressants such as cyclosporin).

This invention will now be described with reference to the following non-limiting Figures and Examples.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Figure 1A shows titer of pooled human serum before and after absorption. Titer obtained by hemagglutination on RBC (hatched bars) and rosetting assay on PBL (open bars) and spleen cells (solid bars). Absorption studies demonstrated that the same xeno antigens were present on all of these tissues (Figure 1 and Figure 2), as absorption with RBC, spleen cells or PBL, removed reactivity for the other cells (Figure 1A and Figure 2). Absorption of the serum pool with EC, while removing all of the EC reactive antibodies (Figure 2A), completely removed all PBL reactive antibodies and almost all the RBC hemagglutinating antibodies (titer fell from 1/128 to 1/2) (Figure 1A). Absorption with RBC removed 75% (Figure 2B) and spleen cells all (Figure 2C) of the endothelial cell (CE) reactive antibodies shown by flow cytometry. Thus, common epitopes are present on pig red cells, PBL, spleen and endothelial cells. Serum absorbed with EC was not tested on PBL or spleen cells. Figure 1B -- see Figure 3.

Figure 2: Testing of pig EC with pooled human serum before and after absorption. In each panel EC tested with absorbed serum (dotted line) or non absorbed serum (solid line). Serum absorbed with EC (panel A), RBC (panel B) or spleen cells (panel C). Binding of human antibody was detected using sheep anti-human IgM and analysis by flow cytometry.

Figure 3: Hemagglutination titer of treated and untreated human serum. Untreated human serum (A); protein-A non binding immunoglobulin (B); protein-A eluted immunoglobulin (C); serum treated with 2-mercaptoethanol (D). Figure 1B shows the same data with the addition of data obtained using a high molecular weight immunoglobulin fraction. Figure 1B: Untreated human serum (A); protein-A non binding immunoglobulin (B); high molecular weight fraction (C); protein-A eluted immunoglobulin (D); serum treated with 2-mercaptoethanol (E).

Figure 4: Carbohydrate inhibition of hemagglutination of normal human serum. Human serum was titrated in the presence of 300mM solutions of carbohydrates.

Figure 5: Concentration of carbohydrate giving 50% inhibition of hemagglutination titer of normal human serum. Only carbohydrates inhibiting hemagglutination in Figure 4 were used in this experiment, with glucose and methyl- β -galactopyranoside as negative controls.

Figure 6: Hemagglutination titer of human serum on pig RBC pre and post absorption on a melibiose column. Human serum was absorbed with equal volumes of melibiose-sepharose (solid bars) or sepharose (open bars), a number of times as indicated in the figure axis.

Figure 7: Southern blot of pig genomic DNA probed with the cDNA insert of clone pPGT-4.

BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

SEQ ID NO:1 Partial nucleotide and predicted amino acid sequence of the pig Gal α (1,3) transferase.

5 SEQ ID NO:2 Complete nucleotide and predicted amino acid sequence of the pig Gal α (1,3) transferase.

SEQ ID NO:3 Nucleotide sequence for PCR primer α GT-1.

SEQ ID NO:4 Nucleotide sequence for PCR primer α GT-2.

10 With regard to SEQ ID NOS:1-2, it should be noted that the present invention is not limited to the specific sequences shown, but, in addition to the mutations discussed above, also includes changes that are found as naturally occurring allelic variants of the porcine Gal
15 α (1,3) galactosyl transferase gene, as well as nucleic acid mutations which do not change the amino acid sequences set forth in these sequences, e.g., third nucleotide changes in degenerate codons.

EXAMPLE 120 Materials and Methods

Cells. Pig cells and tissues were obtained from an abattoir from freshly slaughtered animals. Whole blood was centrifuged at 800g, and erythrocytes (RBC) obtained and were washed three times in phosphate buffered saline
25 (PBS); pig peripheral blood lymphocytes (PBL) were isolated by density gradient centrifugation using ISOPAQUE FICOLL (Vaughan et al, (1983) Transplantation 36:446-450). Pig splenocytes were obtained from whole

spleen by teasing tissue through a sieve to give a single cell suspension. Endothelial cell (EC) cultures were established after treatment of sterile pig aorta with Collagenase Type 4 (Worthington Biochemical Corporation, New Jersey) and the isolated cells were grown in Dulbecco's modified Eagles medium (DMEM) (ICN Biomedicals Australasia Pty Ltd, Seven Hills, NSW) on gelatin coated plates at 37°C. The endothelial origin of EC cultures was verified using rabbit anti human von Willebrand factor antibody (Dako A/S, Copenhagen) and indirect immunofluorescence. COS cells used were maintained in fully supplemented DMEM medium.

Antibodies. Human serum was obtained from a panel of normal volunteers, heat inactivated and pooled before use. The mAb HuLy-m3 (CD48), was used as a negative control (Vaughan Supra). Equal volumes of human serum and 5 to 200mM 2-mercaptoethanol were incubated at 37°C for one hour to destroy IgM.

Absorptions. Pooled serum was absorbed with equal volumes of washed, packed cells for 15 minutes at 37°C, for 15 minutes at 4°C, serum obtained and the procedure repeated three times. For the absorption with melibiose-agarose (Sigma, St Louis, MO) and sepharoseTM (Pharmacia LKB Biotechnology, Uppsala, Sweden), equal volumes packed beads and serum were incubated at 37°C for 16 hours, the beads removed by centrifugation, and the absorption repeated several times.

Serological Assays. a) Hemagglutination: 50 μ l of 0.1% pig RBC were added to 50 μ l of human serum in 96 well plates, incubated at 37°C for 30 minutes, room temperature for 30 minutes and on ice for 60 minutes prior to both macroscopic and microscopic evaluation of hemagglutination; b) Rosetting: Sheep anti human IgG was coupled to sheep RBC with chromic chloride and used in a rosetting assay (Parish et al (1978) J Immunol. Methods 20:173-183); c) Cytofluorographic analysis was performed on FACScanTM (Becton Dickinson, San Jose, CA) (Vaughan et al (1991) Immunogenetics 33:113-117); d) Indirect immunofluorescence was performed on cell monolayers in 6 well tissue culture plates using fluoresceinated sheep anti human IgM or IgG (Silenus Laboratories Pty Ltd, Hawthorn, Victoria, Australia) (Vaughan Supra).

Sugar Inhibitions. Two types of sugar inhibition assays were performed: a) 50 μ l of sugars (300mM in PBS) were added to 50 μ l of doubling dilutions of human serum in 96 well plates, incubated overnight at 4°C and then 50 μ l of 0.1% pig RBC added and the hemagglutination assay performed; b) Human serum, diluted in PBS at one dilution less than that of the 50% hemagglutination titer, was added to 50 μ l of doubling dilutions of sugars (starting at 300mM) and incubated overnight at 4°C, after which 50 μ l of 0.1% pig RBC was added and the hemagglutination assay performed.

Murine Gal α (1-3) Transferase cDNA construct. A cDNA clone, encoding the mouse α (1,3)galactosyl

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transferase was produced using the published sequence of this transferase (Larsen et al (1989) J Biol. Chem 264:14290-14297) and the polymerase chain reaction (PCR) technique. Briefly two oligonucleotides were synthesized; α GT-1 (5'-GAATTCAAGC TTATGATCAC TATGCTTCAA G-3') which is the sense oligonucleotide encoding the first six amino acids of the mature α GT and contains a HindIII restriction site, and α GT-2 (5'-GAATTCCTGC AGTCAGACAT TATTCTAAC-3') which is the anti-sense oligonucleotide encoding the last 5 amino acids of the mature α GT and the in phase termination codon and contains a PstI restriction site. This oligonucleotide pair was used to amplify a 1185 bp fragment from a C57BL/6 spleen cell cDNA library (Sandrin et al (1992) J Immunol. 194:1636-1641). The 1185 bp fragment was purified from a Low Gelling point agarose gel, digested with HindIII and PstI (Pharmacia) restriction endonucleases, and directionally cloned into HindIII/PstI digested CDM8 vector (Seed B (1987) Nature 329:840 842) using T4 ligase (Pharmacia). The product of the ligation was used to transform MC1061/p3, and DNA prepared from resultant colonies for further examination. One plasmid (p α GT-3) having the 1185 bp fragment was selected for further studies. Plasmid DNA was prepared, sequenced to confirm the correct DNA sequence, and used for COS cells transfection experiments using DEAE/Dextran (Vaughan et al (1991) Immunogenetics 33: 113-117; Sandrin et al

(1992) J Immunol. 194:1636-1641, Seed B (1987) Nature 329:840-842).

EXAMPLE 2

Human Anti-pig Antibodies Detect

Epitopes Present on Different Cells

To establish that human serum contains antibodies to pig cells which are predominantly of the IgM class, a pool of human serum was made (from 10 donors) and found to contain antibodies which reacted with pig red cells (by hemagglutination); pig lymphocytes (rosetting assay and flow cytometry); pig spleen cells (rosetting); and pig endothelial cells (flow cytometry) (Figures 1 and 2). Absorption studies demonstrated that the same xeno antigens were present on all of these tissues (Figure 1 and Figure 2), as absorption with RBC, spleen cells or PBL, removed reactivity for the other cells (Figure 1A and Figure 2). Absorption of the serum pool with EC, while removing all of the EC reactive antibodies (Figure 2a), completely removed all PBL reactive antibodies and almost all the RBC hemagglutinating antibodies (titer fell from 1/128 to 1/2) (Figure 1A). Absorption with RBC removed 75% (Figure 2B) and spleen cells all (Figure 2C) of the EC reactive antibodies shown by flow cytometry. Thus, common epitopes are present on pig red cells, PBL, spleen and endothelial cells.

Most of the activity in the serum pool was due to IgM rather than IgG antibodies as indicated by the inability of a protein A-sepharose column, which does not

bind IgM, to alter the titer of the serum after passage through the column (Figure 3), and IgG antibodies eluted from the protein A-sepharose column reacted only weakly with RBC (Figure 3). Furthermore, treatment of the serum with 2-mercaptoethanol, which destroys IgM but leaves IgG intact, led to a complete loss of antibody activity (Figure 3). When the serum was fractionated by SEPHACRYL™ gel chromatography, the high molecular weight fractions (IgM) were reactive with RBC, whereas the low molecular weight fractions (IgG) were not (data not shown). Thus the different pig cells carry similar epitopes, all reacted with IgM antibodies and in our assays there was little IgG activity found in the human serum for pig cells.

15

EXAMPLE 3

Human Anti-pig Antibodies React Predominantly With Terminal Galactose Residues

The ability of different carbohydrates to inhibit the hemagglutination reaction (Figure 4) was examined. Of the sugars tested, inhibition as measured by a decrease in titer, was observed with 300mM galactose, methyl- α -D-galactopyranoside, melibiose and stachyose, all of which decreased the titer of the serum pool by 75% (Figure 4); and with 300mM D-galactosamine, for which a 50% decrease in titer was observed (Figure 4). None of the other monosaccharides tested (listed in the figure legend) had any effect on hemagglutination titer (Figure 4). These studies demonstrated that galactose is the

part of the epitope, as both melibiose and stachyose have terminal galactose residues. It is of interest to note the difference in the ability of galactose in the α (methyl- α -D-galactopyranoside, melibiose and stachyose) but not β (methyl- β -D-galactopyranoside) configuration to inhibit the serum.

The relative avidity of the antibodies for the sugars which inhibited agglutination was estimated from the concentration of sugar giving 50% inhibition of the agglutination titer (Figure 5). Both D-galactose and melibiose achieved this inhibition at <1.5mM, stachyose and methyl- α -D-galactopyranoside at 4.7mM and D-galactosamine at 18.7mM (Figure 5). By contrast, D-glucose and methyl- β -D-galactopyranoside had no effect even at 300mM concentration. Thus D-galactose is an important part of the epitope, as it is a potent inhibitor of the xenoantibodies at low concentration (<1.1 5mM); the ability of methyl- α -D-galactopyranoside to inhibit agglutination at low concentrations (<1.15mM), compared with the failure of methyl- β -D-galactopyranoside (300mM) to inhibit, demonstrates that the galactose residue (which is likely to be a terminal sugar) is in an α -linkage rather than a β -linkage with the subterminal residue. The results obtained with melibiose (Gal α (1,6)Glc) and stachyose (Gal α (1,6)Gal α (1,6)Glc β (1,2)Fru), which have α -linked terminal galactose residues, are in accord with this

conclusion. The inhibition of hemagglutination observed with galactosamine, which has an additional amine side chain on galactose, (50% inhibition of titer at 18.7mM) could be due to a second carbohydrate involved in the epitope, or a lower affinity of the xenoantibodies for this sugar.

To further examine the reaction with galactose, the serum pool was absorbed four times with equal volumes of packed melibiose sepharose or with sepharose as the control (Figure 6), one absorption with melibiose-sepharose decreased the titer of the antibody from 1/32 to 1/4, and two sequential absorptions decreased the titer further to 1/2 (Figure 6). This absorption was specific for melibiose, as using sepharose beads had no effect (Figure 6). Thus the majority of the antibody (=94%) reactive with xenoantigens reacts with galactose in an α -linkage.

EXAMPLE 4

Human Anti-Pig Antibodies React with COS Cells

After Transfection with $\alpha(1,3)$ Galactosyl Transferase

The cDNA coding for the $\alpha(1,3)$ galactosyl transferase which transfers a terminal galactose residue with an $\alpha(1,3)$ linkage to a subterminal galactose has been cloned for both mouse (Larsen et al (1989) J Biol Chem 264:14290-14297) and ox (Joziassse et al (1989) J Biol Chem 264:14290-14297). Using this data we used transfection experiments to determine the role of the Gal $\alpha(1,3)$ Gal epitope in isolation of others. The mouse

transferase was isolated from a cDNA library using the PCR technique, and the PCR product was directionally cloned into the CDM8 vector for expression studies in COS cells. The cDNA insert was sequenced in both directions and shown to be identical to the published nucleotide sequence (Larsen et al (1989) J Biol Chem 264:14290-14297). COS cells, derived from Old World Monkeys, were chosen as they do not react with human serum nor with the IB-4 lectin (which is specific for the Gal α (1,3)Gal epitope) (Table 1). After transfection of COS cells with the α (1,3)galactosyl transferase, the Gal α (1,3)Gal epitope was detected on the cell surface by binding of the IB-4 lectin (Table 1); these cells were also strongly reactive with the serum pool. Absorbing the human sera with pig RBC removed the reactivity for Gal α (1,3)Gal⁺COS cells, (Table 1). Passage of the serum over a protein-A sepharose column had no effect on the reactivity of the serum for Gal α (1,3)Gal⁺COS cells, when using an FITC conjugated sheep anti-human IgM as the second antibody (this was reflected in the same number of reactive cells, the intensity of staining and the titer of the serum (Table 1)). In contrast to this, eluted antibodies reacted only weakly with the Gal α (1,3)Gal⁺COS cells, and this reaction was only observed when using FITC conjugated sheep anti-human IgG or FITC conjugated sheep anti-human Ig, but not FITC conjugated sheep anti-human IgM (Table 1). Thus human serum has IgM antibodies to the Gal α (1,3)Gal epitope which was expressed on

Gal α (1,3)Gal⁺COS cells. The reaction of the serum with Gal α (1,3)Gal⁺COS cells is specific and not due to the transfection procedure as CD48⁺ COS cells were not reactive with either the serum nor the IB-4 lectin (Table 1). Furthermore, the reactivity for both pig RBC (as detected by hemagglutination) and EC (as detected by FACS analysis) could be removed by absorption with Gal α (1,3)Gal⁺COS cells but not untransfected COS cells. Thus human serum pool contains IgM antibodies reactive with the Gal α (1,3)Gal epitope.

The level of antibodies in human serum reactive with the Gal α (1,3)Gal epitope can be used to determine the propensity of a patient to hyperacutely reject a porcine xenotransplant. In addition, the level of such antibodies can be used to determine the amount of antibody antagonist that should be administered to a patient prior to such xenotransplantation.

The level of these antibodies can be effectively determined using the transfected and untransfected COS cells described above as matched Gal α (1,3)Gal⁺ and Gal α (1,3)Gal⁻ absorbants, followed by a measurement of the reactivity of the absorbed serum for pig RBC and/or EC. Higher levels of serum antibody will result in a larger difference in reactivity of the serum absorbed against the Gal α (1,3)Gal⁺ absorbant versus that absorbed against the Gal α (1,3)Gal⁻ absorbant. Cells from other species, e.g., human cells, can be used in such an assay. Also, rather than using a DNA sequence encoding the murine

transferase, a DNA sequence encoding the porcine transferase (see Example 5) can be used. Such a porcine transferase is preferred since there may be differences in the action of the murine and porcine transferases, e.g., altered sensitivity to the macromolecular environment of the galactose substrate of the enzyme, and for a porcine xenotransplantation, it is the level of antibodies against the Gal α (1,3)Gal epitope in the porcine macromolecular environment that is of interest.

In addition to the foregoing, the transfected Gal α (1,3)Gal⁺ cells described above can also be used as absorbants to remove anti-Gal α (1,3)Gal antibodies from human serum, e.g., by binding such cells to a solid support and passing the serum over the immobilized cells.

15

EXAMPLE 5

Cloning of Porcine α (1,3) Galactosyl Transferase

Utilizing the murine cDNA clone for the α (1,3) galactosyl transferase as a hybridization probe we have cloned the pig α (1,3) galactosyl transferase from a λ GT11 pig spleen cDNA library (Clontech Laboratories, Palo Alto, CA) according to standard methods as described in Sambrook et al (supra). This clone, pPGT-4, has been deposited with the AGAL and assigned accession number N94/9030. SEQ ID NO:1 shows a partial nucleotide sequence and predicted amino acid sequence of pig Gal α (1,3) transferase as determined by sequencing of clone pPGT-4. The sequence shown is incomplete at the 5' end.

25

Utilizing the cDNA insert of the pPGT-4 clone as a hybridization probe we have also cloned the 5' end of the pig $\alpha(1,3)$ galactosyl transferase from a 5' STRECH pig liver cDNA library in λ gt10, according to standard methods as described in Sambrook et al (supra). The insert was obtained by the PCR technique using a λ oligonucleotide, and an oligonucleotide made to the pig sequence. This PCR product was subcloned into SmaI cut pBLUESCRIPT KS⁺. This clone, pPGT-2, has been deposited with the AGAL and assigned accession number N94/9029.

SEQ ID NO:2 shows a complete nucleotide sequence and predicted amino acid sequence of pig Gal $\alpha(1,3)$ transferase as determined by sequencing of clones pPGT-4 and pPGT-2. The pig transferase has high sequence homology with both the murine and bovine $\alpha(1,3)$ galactosyl transferase genes.

Both the partial and complete cDNA sequences of SEQ ID NOS:1-2 can be used in the xenotransplant therapies discussed above. For example, using techniques well known in the art, all or a part of any of the nucleotide sequences of SEQ ID NOS:1-2, when inserted into replicating DNA, RNA or DNA/RNA vectors, can be used to reduce the expression of the Gal $\alpha(1,3)$ transferase in porcine cells by directing the expression of anti-sense RNAs in transgenic cells or animals. See, for example, Biotechniques, 6(10):958-976, 1988.

In addition, as illustrated in the following example, the sequences of SEQ ID NOS:1-2 can be used as hybridization probes for the characterization and isolation of genomic clones encoding the porcine Gal α (1,3) transferase. Mutants of the genomic nucleotide sequence, in turn, can be used in homologous recombination techniques of the types described above so that destruction of the functional gene takes place in porcine cells.

EXAMPLE 6

Characterization and Isolation of the Porcine Gene Encoding α (1,3) Galactosyl Transferase

Genomic DNA prepared from pig spleen tissue was digested with EcoRI, BamH1, PstI, HindIII, KpnI and BstEII, electrophoresed on a 0.8% agarose gel and transferred to a nylon filter, the final wash was at 65°C in 0.1x SSC, 0.1% SDS. As shown in Figure 7, the genomic Southern blot demonstrated a simple pattern suggesting that the gene exists as a single copy with a genomic size of ~25kb.

Utilizing the cDNA insert of the pPGT-4 clone as a hybridization probe, we have cloned the porcine α (1,3) galactosyl transferase gene from a pig genomic DNA EMBL library (Clontech Laboratories, Inc., Palo Alto, CA) according to standard methods as described in Sambrook et al (supra). This cloning has resulted in the isolation of two lambda phage clones, λ PGT-g1 and λ PGT-g5 that

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contain different regions of the porcine transferase gene.

As discussed above, the gene for the $\alpha(1,3)$ galactosyl transferase can be used to effect targeted
5 destruction of the native gene for this enzyme using
homologous recombination technology. In accordance with
the conventional techniques used in this art, such gene
knockout is performed using fragments obtained from
genomic clones of the type provided by this example. The
10 gene destruction can be performed in somatic or stem
cells (Capecchi, 1989, supra). Because such genetically
engineered cells do not produce the Gal $\alpha(1,3)$ Gal epitope,
they and their progeny are less likely to induce
hyperacute rejection in humans and are thus suitable for
15 xenotransplantation into human patients.

EXAMPLE 7

Production of Anti-idiotypic Antibodies

Against Human Anti-Gal $\alpha(1,3)$ Gal Antibodies

Polyclonal anti-idiotypic antibodies against human
20 anti-Gal $\alpha(1,3)$ Gal antibodies are prepared following the
procedures of Coligan, et al., 1992, supra; Harlow and
Lane, 1988, supra; and Liddell and Cryer, 1991, supra.
Human anti-Gal $\alpha(1,3)$ Gal antibodies are absorbed from
pooled human serum onto immobilized melibiose (melibiose-
25 sepharose or melibiose-agarose) as described above in
Example 3. The antibodies are eluted using standard
methods, such as, high or low pH, high salt, and/or
chaotropic agents. Fab' fragments are prepared following

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dialysis into an appropriate buffer. The Fab' fragments are used to immunize rabbits, goats, or other suitable animals, along with conventional adjuvants.

5 The resulting polyclonal antisera are tested for their ability to change the conformation of the human antibody reactive site so as to reduce its affinity for the Gal α (1,3)Gal epitope. Those sera that produce such reduced affinity constitute the desired anti-idiotypic antibodies.

10 Monoclonal antibodies are produced using the same Fab' fragments as antigens to immunize appropriate strains of mice. Hybridomas are prepared by fusing spleen cells from such immunized mice with murine myeloma cells. Supernatants are tested for antibodies having the
15 ability to change the conformation of the human antibody reactive site so as to reduce its affinity for the Gal α (1,3)Gal epitope. Those antibodies that produce such reduced affinity constitute the desired monoclonal anti-idiotypic antibodies.

20 The finding that the majority of xenoreactive IgM is directed to the enzymatic product of the single transferase raises the possibility of producing transgenic pigs lacking the epitope, by targeted destruction of the α (1,3) galactosyl transferase genes
25 using homologous recombination technology. Such genetically modified pigs could be used for transplantation. The destruction of the gene is likely to have no deleterious effect on the pig - humans live

normally in its absence.

This invention has been described by way of example only and is in no way limited by the specific examples herewith.

5

DEPOSITS

Clones pPGT-4, pPGT-2, λ PGT-g1, and λ PGT-g5, discussed above, have been deposited with the Australian Government Analytical Laboratories, (AGAL), 1 Suakin Street, Pymble, N.S.W. 2073, Australia, and have been assigned the designations N94/9030, N94/9029, N94/9027, and N94/9028, respectively. These deposits were made under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure (1977). These deposits were made on March 11, 1994.

10

15

TABLE 1Serology On Transfected COS Cells

| <u>Serum</u> | <u>Target</u> | <u>Reaction¹</u> |
|----------------------|-----------------------|-----------------------------|
| NHS | GT ⁺ COS | +++ |
| NHS abs RBC | GT ⁺ COS | - |
| NHS Tx 2-ME | GT ⁺ COS | - |
| NHS abs Protein A | GT ⁺ COS | +++ ² |
| NHS Eluted Protein A | GT ⁺ COS | + ³ |
| CD48 | GT ⁺ COS | - |
| NHS | CD48 ⁺ COS | - |
| CD48 | CD48 ⁺ COS | +++ |
| NHS | COS | - |
| CD48 | COS | - |
| <hr/> | | |
| IB4 ⁴ | GT ⁺ COS | +++ |
| IB4 | CD48 ⁺ COS | - |
| IB4 | COS | - |

¹ Reactivity detected by indirect immunofluorescence using FITC conjugated sheep anti-human Ig or FITC conjugated sheep anti-mouse Ig unless otherwise stated.

² No difference in titer was observed when tested with FITC conjugated sheep anti-human IgM.

³ Reaction detected on protein A purified immunoglobulin using FITC conjugated sheep anti-human Ig or FITC conjugated sheep anti-human IgG, but not with FITC conjugated sheep anti-human IgM.

⁴ Reactivity detected using FITC conjugated IB4 lectin.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: The Austin Research Institute
- (ii) TITLE OF INVENTION: XENOTRANSPLANTATION THERAPIES
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Peter A. Stearne
 - (B) STREET: Level 10, 10 Barrack Street
 - (C) CITY: Sydney
 - (D) STATE: New South Wales
 - (E) COUNTRY: Australia
 - (F) Postal Code 2001
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Peter A. Stearne
 - (C) REFERENCE/DOCKET NUMBER: 462552/pas
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 612 262 2611
 - (B) TELEFAX: 612 262 1080

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1353 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to mRNA

(A) DESCRIPTION: galactosyl transferase, 3' clone

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Sus scrofa

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

| | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| GTA | CCG | AGC | TCG | AAT | TCC | GCA | AGC | CAG | TCA | CCA | CAA | GCC | ATG | 42 |
| Val | Pro | Ser | Ser | Asn | Ser | Ala | Ser | Gln | Ser | Pro | Gln | Ala | Met | |
| | | | 50 | | | | | 55 | | | | | 60 | |
| ACT | GAC | CCA | TGT | TCC | CCC | AGA | CTG | TCG | TAC | CTT | AGC | AAA | GCC | 84 |
| Thr | Asp | Pro | Cys | Ser | Pro | Arg | Leu | Ser | Tyr | Leu | Ser | Lys | Ala | |
| | | | | 65 | | | | 70 | | | | | | |
| ATC | CTG | ACT | CTA | TGT | TTT | GTC | ACC | AGG | AAA | CCC | CCA | GAG | GTC | 126 |
| Ile | Leu | Thr | Leu | Cys | Phe | Val | Thr | Arg | Lys | Pro | Pro | Glu | Val | |
| | 75 | | | | 80 | | | | | 85 | | | | |
| GTG | ACC | ATA | ACC | AGA | TGG | AAG | GCT | CCA | GTG | GTA | TGG | GAA | GGC | 168 |
| Val | Thr | Ile | Thr | Arg | Trp | Lys | Ala | Pro | Val | Val | Trp | Glu | Gly | |
| | 90 | | | | | 95 | | | | | 100 | | | |
| ACT | TAC | AAC | AGA | GCC | GTC | TTA | GAT | AAT | TAT | TAT | GCC | AAA | CAG | 210 |
| Thr | Tyr | Asn | Arg | Ala | Val | Leu | Asp | Asn | Tyr | Tyr | Ala | Lys | Gln | |
| | | 105 | | | | | 110 | | | | | 115 | | |
| AAA | ATT | ACC | GTG | GGC | TTG | ACG | GTT | TTT | GCT | GTC | GGA | AGA | TAC | 252 |
| Lys | Ile | Thr | Val | Gly | Leu | Thr | Val | Phe | Ala | Val | Gly | Arg | Tyr | |
| | | | 120 | | | | | 125 | | | | | 130 | |
| ATT | GAG | CAT | TAC | TTG | GAG | GAG | TTC | TTA | ATA | TCT | GCA | AAT | ACA | 294 |
| Ile | Glu | His | Tyr | Leu | Glu | Glu | Phe | Leu | Ile | Ser | Ala | Asn | Thr | |
| | | | | 135 | | | | | 140 | | | | | |
| TAC | TTC | ATG | GTT | GGC | CAC | AAA | GTC | ATC | TTT | TAC | ATC | ATG | GTG | 336 |
| Tyr | Phe | Met | Val | Gly | His | Lys | Val | Ile | Phe | Tyr | Ile | Met | Val | |
| | 145 | | | | 150 | | | | | 155 | | | | |
| GAC | GAT | ATC | TCC | AGG | ATG | CCT | TTG | ATA | GAG | CTG | GGT | CCT | CTG | 378 |
| Asp | Asp | Ile | Ser | Arg | Met | Pro | Leu | Ile | Glu | Leu | Gly | Pro | Leu | |
| | 160 | | | | | 165 | | | | | 170 | | | |
| CGT | TCC | TTT | AAA | GTG | TTT | GAG | ATC | AAG | TCC | GAG | AAG | AGG | TGG | 420 |
| Arg | Ser | Phe | Lys | Val | Phe | Glu | Ile | Lys | Ser | Glu | Lys | Arg | Trp | |
| | | 175 | | | | | 180 | | | | | 185 | | |
| CAA | GAC | ATC | AGC | ATG | ATG | CGC | ATG | AAG | ACC | ATC | GGG | GAG | CAC | 462 |
| Gln | Asp | Ile | Ser | Met | Met | Arg | Met | Lys | Thr | Ile | Gly | Glu | His | |
| | | | 190 | | | | | 195 | | | | | 200 | |
| ATC | CTG | GCC | CAC | ATC | CAG | CAC | GAG | GTG | GAC | TTC | CTC | TTC | TGC | 504 |
| Ile | Leu | Ala | His | Ile | Gln | His | Glu | Val | Asp | Phe | Leu | Phe | Cys | |
| | | | | 205 | | | | | 210 | | | | | |

| | | | | | | | | | | | | | |
|-------------------|-------------------|-------------------|-------------------|-------------------|--------------------------|--------------------------|--------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| ATT Ile 215 | GAC Asp Val | GTG Val 220 | GAT Asp Gln | CAG Gln Val | GTC Val 220 | TTC Phe Gln | CAA Gln Asn | AAC Asn Asn | AAC Phe Gly | TTT Phe Gly | GGG Gly Val | GTG Val Glu | GAG Glu 246 |
| ACC Thr 230 | CTG Leu 230 | GGC Gly Gln | CAG Gln Ser | TCG Ser Val | GTC Val 325 | GCT Ala Gln | CAG Gln Leu | CTA Leu Gln | CAG Gln Ala | GCC Ala Trp | TGG Trp Tyr | TGG Tyr 240 | TAC Tyr 240 |
| AAG Lys 245 | GCA Ala His | CAT His Pro | CCT Pro Asp | GAC Asp Glu | GAG Glu Phe | TTC Phe Thr | ACC Thr Tyr | TAC Tyr Glu | GAG Glu Arg | CGG Arg Pro | CCG Pro Lys | AAG Lys Glu | GAG Glu 255 |
| TCC Ser 260 | GCA Ala Ala | GCC Ala Tyr | TAC Tyr Ile | ATT Ile Pro | CCG Pro Phe | TTT Phe Arg | CGC Arg Gln | CAG Gln Gly | GGG Gly Asp | GAT Asp Phe | TTT Phe Tyr | TAT Tyr Tyr | TAC Tyr 270 |
| CAC His 275 | GCA Ala Ala | GCC Ala Ile | ATT Ile Leu | TTG Leu Gly | GGG Gly Gly | GGA Gly Thr | ACA Thr Pro | CCC Pro Thr | ACT Thr Gln | CAG Gln Val | GTT Val Leu | CTA Leu Asn | AAC Asn 280 |
| ATC Ile 285 | ACT Thr Gln | CAG Gln Glu | GAG Glu Cys | TGC Cys Phe | TTC Phe Lys | AAG Lys Gly | GGA Gly Ile | ATC Ile Leu | CTC Leu Gln | CAG Gln Asp | GAC Asp Lys | AAG Lys Glu | GAA Glu 295 |
| AAT Asn 300 | GAC Asp Ile | ATA Ile Glu | GAA Glu Ala | GCC Ala Glu | GAG Glu Trp | TGG Trp His | CAT His Asp | GAT Asp Glu | GAA Glu Ser | AGC Ser Gly | GGG Gly Leu | CTA Leu Asn | AAC Asn 310 |
| AAG Lys 315 | TAT Tyr Phe | TTC Phe Leu | CTT Leu Leu | CTC Leu Asn | AAC Asn Lys | AAA Lys Pro | CCC Pro Thr | ACT Thr Lys | AAA Lys Ile | ATC Ile Leu | TTA Leu Ser | TCC Ser Pro | CCA Pro 325 |
| GAA Glu 330 | TAC Tyr Cys | TGC Cys Trp | TGG Trp Asp | GAT Asp Tyr | TAT Tyr His | CAT His Ile | ATA Ile Gly | GGC Gly Met | ATG Met Ser | TCT Ser Val | GTG Val Asp | GAT Asp Ile | ATT Ile 340 |
| AGG Arg 345 | ATT Ile Val | GTC Val Lys | AAG Lys Gly | GGG Gly Ala | GCT Ala Trp | TGG Trp Gln | CAG Gln Lys | AAA Lys Lys | AAA Lys Glu | GAG Glu Tyr | TAT Tyr Asn | AAT Asn Leu | TTG Leu 350 |
| GTT Val 355 | AGA Arg Asn | AAT Asn Asn | AAC Asn Ile | ATC Ile Val | TGACTTTAAA Val 355 | TTGTGCCAGC Val 355 | AGTTTTCTGA Val 355 | | | | | | |
| ATTGAAAGA | GTATTACTCT | GGCTACTTCC | TCAGAGAAGT | AGCACTTAAT | 1019 | | | | | | | | |
| TTTAACTTTT | CAAAAATAC | TAACAAAATA | CCAACACAGT | AAGTACATAT | 1069 | | | | | | | | |
| TATTCTTCCT | TGCAACTTTG | AGCCTTGTC | AATGGGAGAA | TGACTCTGTA | 1119 | | | | | | | | |
| GTAATCAGAT | GTAAATTCCC | AATGATTCT | TATCTGCGGA | ATTCCAGCTG | 1169 | | | | | | | | |
| AGCGCCGGTC | CTACCATTAC | CAGTTGGTCT | GGTGTGACG | ACTCCTGGAG | 1219 | | | | | | | | |
| CCCGTCAGTA | TCGGCGGAAT | TCGCGGCCGG | GCGCCAATGC | ATTGGGCCCA | 1269 | | | | | | | | |

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ATTCCGCCCT ATAGTGAGTC GTATTACAAT TCACTGGCCG TGTTTTACAA 1319

CCTCGTGACT GGGAAAACCC TGGCCTTACC CAAC 1353

SUBSTITUTE SHEET (Rule 26)

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1423 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to mRNA

- (A) DESCRIPTION: galactosyl transferase,
full coding sequence

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Sus scrofa

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

| | |
|--|-----|
| CGGGGGCCAT CCCCAGCGC ACCCAGCTTC TGCCGATCAG GAGAAAATA | 49 |
| ATG AAT GTC AAA GGA AGA GTG GTT CTG TCA ATG CTG CTT GTC Met Asn Val Lys Gly Arg Val Val Leu Ser Met Leu Leu Val | 91 |
| 5 10 | |
| TCA ACT GTA ATG GTT GTG TTT TGG GAA TAC ATC AAC AGA AAC Ser Thr Val Met Val Val Phe Trp Glu Tyr Ile Asn Arg Asn | 133 |
| 15 20 25 | |
| CCA GAA GTT GGC AGC AGT GCT CAG AGG GGC TGG TGG TTT CCG Pro Glu Val Gly Ser Ser Ala Gln Arg Gly Trp Trp Phe Pro | 175 |
| 30 35 40 | |
| AGC TGG TTT AAC AAT GGG ACT CAC AGT TAC CAC GAA GAA GAA Ser Trp Phe Asn Asn Gly Thr His Ser Tyr His Glu Glu Glu | 217 |
| 45 50 55 | |
| GAC GCT ATA GGC AAC GAA AAG GAA CAA AGA AAA GAA GAC AAC Asp Ala Ile Gly Asn Glu Lys Glu Gln Arg Lys Glu Asp Asn | 259 |
| 60 65 70 | |
| AGA GGA GAG CTT CCG CTA GTG GAC TGG TTT AAT CCT GAG AAA Arg Gly Glu Leu Pro Leu Val Asp Trp Phe Asn Pro Glu Lys | 301 |
| 75 80 | |
| CGC CCA GAG GTC GTG ACC ATA ACC AGA TGG AAG GCT CCA GTG Arg Pro Glu Val Val Thr Ile Thr Arg Trp Lys Ala Pro Val | 343 |
| 85 90 95 | |
| GTA TGG GAA GGC ACT TAC AAC AGA GCC GTC TTA GAT AAT TAT Val Trp Glu Gly Thr Tyr Asn Arg Ala Val Leu Asp Asn Tyr | 385 |
| 100 105 110 | |
| TAT GCC AAA CAG AAA ATT ACC GTG GGC TTG ACG GTT TTT GCT Tyr Ala Lys Gln Lys Ile Thr Val Gly Leu Thr Val Phe Ala | 427 |
| 115 120 125 | |
| GTC GGA AGA TAC ATT GAG CAT TAC TTG GAG GAG TTC TTA ATA Val Gly Arg Tyr Ile Glu His Tyr Leu Glu Glu Phe Leu Ile | 469 |
| 130 135 140 | |
| TCT GCA AAT ACA TAC TTC ATG GTT GGC CAC AAA GTC ATC TTT Ser Ala Asn Thr Tyr Phe Met Val Gly His Lys Val Ile Phe | 511 |
| 145 150 | |
| TAC ATC ATG GTG GAT GAT ATC TCC AGG ATG CCT TTG ATA GAG Tyr Ile Met Val Asp Asp Ile Ser Arg Met Pro Leu Ile Glu | 553 |
| 155 160 165 | |

| | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|------------|-----|-----|-----|-----|------|
| CTG | GGT | CCT | CTG | CGT | TCC | TTT | AAA | GTG | TTT | GAG | ATC | AAG | TCC | 595 |
| Leu | Gly | Pro | Leu | Arg | Ser | Phe | Lys | Val | Phe | Glu | Ile | Lys | Ser | |
| 170 | | | | | | 175 | | | | | 180 | | | |
| GAG | AAG | AGG | TGG | CAA | GAC | ATC | AGC | ATG | ATG | CGC | ATG | AAG | ACC | 637 |
| Glu | Lys | Arg | Trp | Gln | Asp | Ile | Ser | Met | Met | Arg | Met | Lys | Thr | |
| | 185 | | | | | 190 | | | | | | 195 | | |
| ATC | GGG | GAG | CAC | ATC | CTG | GCC | CAC | ATC | CAG | CAC | GAG | GTG | GAC | 679 |
| Ile | Gly | Glu | His | Ile | Leu | Ala | His | Ile | Gln | His | Glu | Val | Asp | |
| | | | 200 | | | | | 205 | | | | | 210 | |
| TTC | CTC | TTC | TGC | ATT | GAC | GTG | GAT | CAG | GTC | TTC | CAA | AAC | AAC | 721 |
| Phe | Leu | Phe | Cys | Ile | Asp | Val | Asp | Gln | Val | Phe | Gln | Asn | Asn | |
| | | | | 215 | | | | | 220 | | | | | |
| TTT | GGG | GTG | GAG | ACC | CTG | GGC | CAG | TCG | GTG | GCT | CAG | CTA | CAG | 763 |
| Phe | Gly | Val | Glu | Thr | Leu | Gly | Gln | Ser | Val | Ala | Gln | Leu | Gln | |
| 225 | | | | | 230 | | | | | 235 | | | | |
| GCC | TGG | TGG | TAC | AAG | GCA | CAT | CCT | GAC | GAG | TTC | ACC | TAC | GAG | 805 |
| Ala | Trp | Trp | Tyr | Lys | Ala | His | Pro | Asp | Glu | Phe | Thr | Tyr | Glu | |
| | 240 | | | | | 245 | | | | | 250 | | | |
| AGG | CGG | AAG | GAG | TCC | GCA | GCC | TAC | ATT | CCG | TTT | GGC | CAG | GGG | 847 |
| Arg | Arg | Lys | Glu | Ser | Ala | Ala | Tyr | Ile | Pro | Phe | Gly | Gln | Gly | |
| | | 255 | | | | | 260 | | | | | 265 | | |
| GAT | TTT | TAT | TAC | CAC | GCA | GCC | ATT | TTT | GGG | GGA | ACA | CCC | ACT | 889 |
| Asp | Phe | Tyr | Tyr | His | Ala | Ala | Ile | Phe | Gly | Gly | Thr | Pro | Thr | |
| | | | 270 | | | | | 275 | | | | | 280 | |
| CAG | GTT | CTA | AAC | ATC | ACT | CAG | GAG | TGC | TTC | AAG | GGA | ATC | CTC | 931 |
| Gln | Val | Leu | Asn | Ile | Thr | Gln | Glu | Cys | Phe | Lys | Gly | Ile | Leu | |
| | | | | 285 | | | | | 290 | | | | | |
| CAG | GAC | AAG | GAA | AAT | GAC | ATA | GAA | GCC | GAG | TGG | CAT | GAT | GAA | 973 |
| Gln | Asp | Lys | Glu | Asn | Asp | Ile | Glu | Ala | Glu | Trp | His | Asp | Glu | |
| 295 | | | | | 300 | | | | | 305 | | | | |
| AGC | CAT | CTA | AAC | AAG | TAT | TTC | CTT | CTC | AAC | AAA | CCC | ACT | AAA | 1015 |
| Ser | His | Leu | Asn | Lys | Tyr | Phe | Leu | Leu | Asn | Lys | Pro | Thr | Lys | |
| | 310 | | | | | 315 | | | | | 320 | | | |
| ATC | TTA | TCC | CCA | GAA | TAC | TGC | TGG | GAT | TAT | CAT | ATA | GGC | ATG | 1057 |
| Ile | Leu | Ser | Pro | Glu | Tyr | Cys | Trp | Asp | Tyr | His | Ile | Gly | Met | |
| | | 325 | | | | | 330 | | | | | 335 | | |
| TCT | GTG | GAT | ATT | AGG | ATT | GTC | AAG | ATA | GCT | TGG | CAG | AAA | AAA | 1099 |
| Ser | Val | Asp | Ile | Arg | Ile | Val | Lys | Ile | Ala | Trp | Gln | Lys | Lys | |
| | | | 340 | | | | 345 | | | | | | 350 | |
| GAG | TAT | AAT | TTG | GTT | AGA | AAT | AAC | ATC | TGACTTTAAA | | | | | 1136 |
| Glu | Tyr | Asn | Leu | Val | Arg | Asn | Asn | Ile | | | | | | |
| | | | | 355 | | | | | | | | | | |

| | |
|--|------|
| TTGTGCCAGC AGTTTTCTGA ATTTGAAAGA GTATTACTCT GGCTACTTCC | 1186 |
| TCAGAGAAGT AGCACTTAAT TTTAACTTTT AAAAAAATAC TAACAAAATA | 1236 |
| CCAACACAGT AAGTACATAT TATTCTTCCT TGCAACTTTG AGCCTTGTC | 1286 |
| AATGGGAGAA TGACTCTGTA GTAATCAGAT GTAAATTCCC AATGATTTCT | 1336 |
| TATCTGCGGA ATTCCAGCTG AGCGCCGGTC GCTACCATTA CCAGTTGGTC | 1386 |
| TGGTGTGAC GACTCCTGGA GCCCGTCAGT ATCGGCG | 1423 |

- (2) INFORMATION FOR SEQ ID NO:3:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 30 bases
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Other Nucleic Acid
- (A) DESCRIPTION: PCR primer α GT-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATTCAAGC TTATGATCAC TATGCTTCAA

30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 bases
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other Nucleic Acid

- (A) DESCRIPTION: PCR primer α GT-2

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

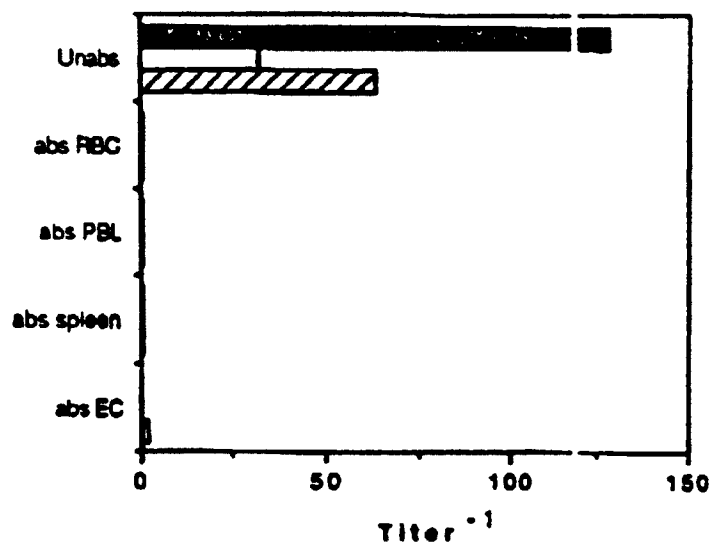
GAATTCCTGC AGTCAGACAT TATTCTAAC

29

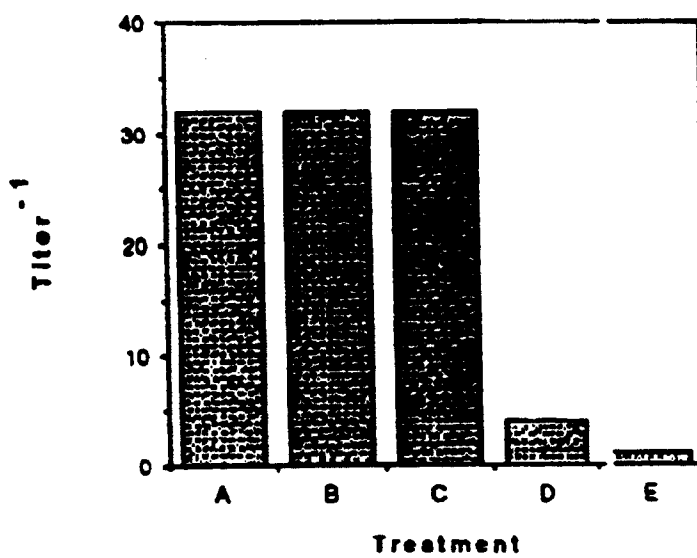
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FIGURE 1

A



B



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FIGURE 2

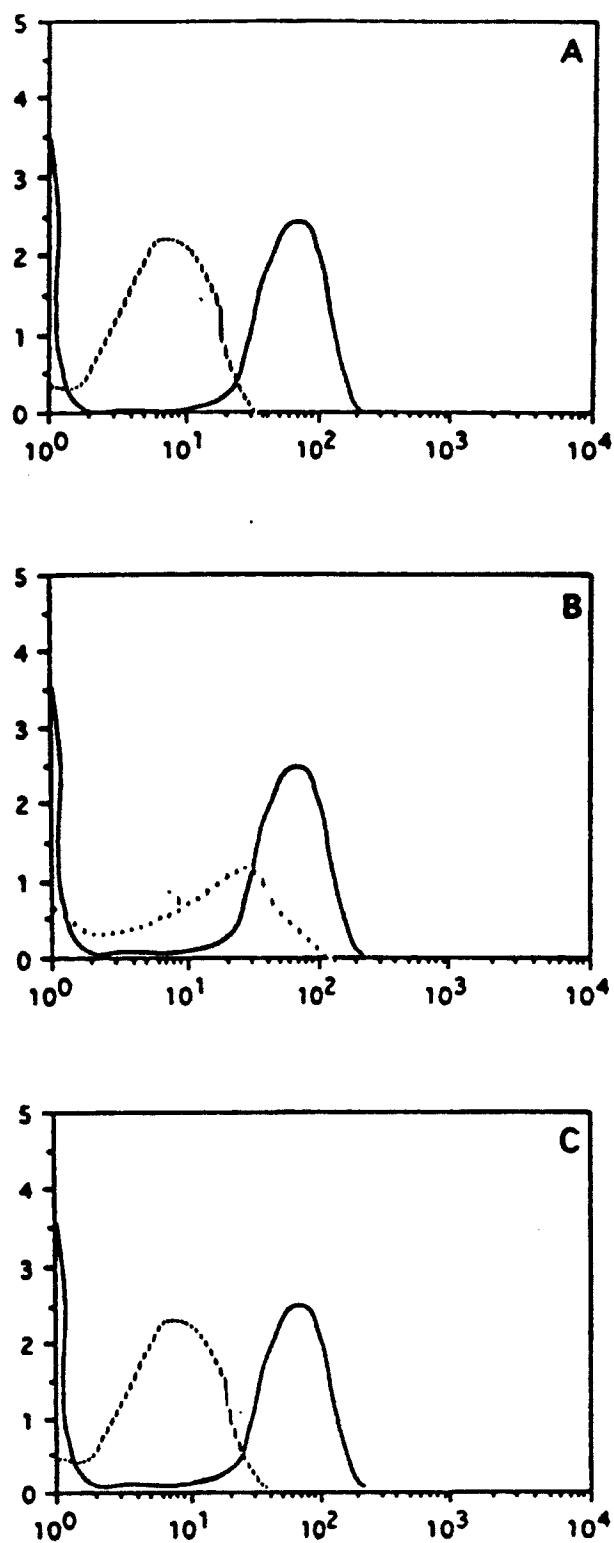


FIGURE 3

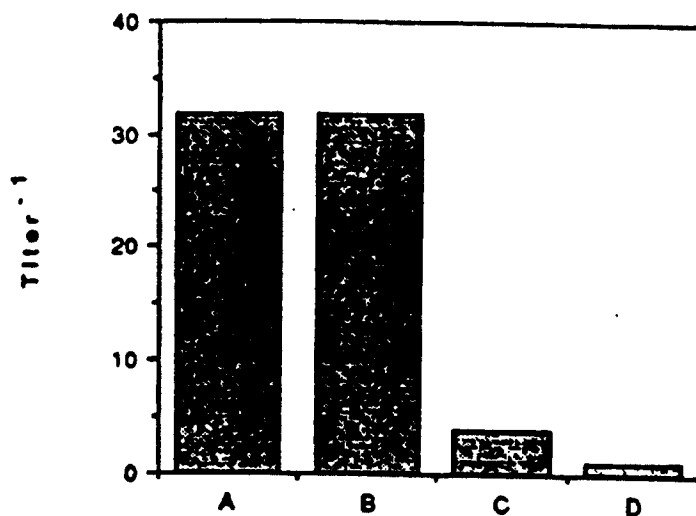
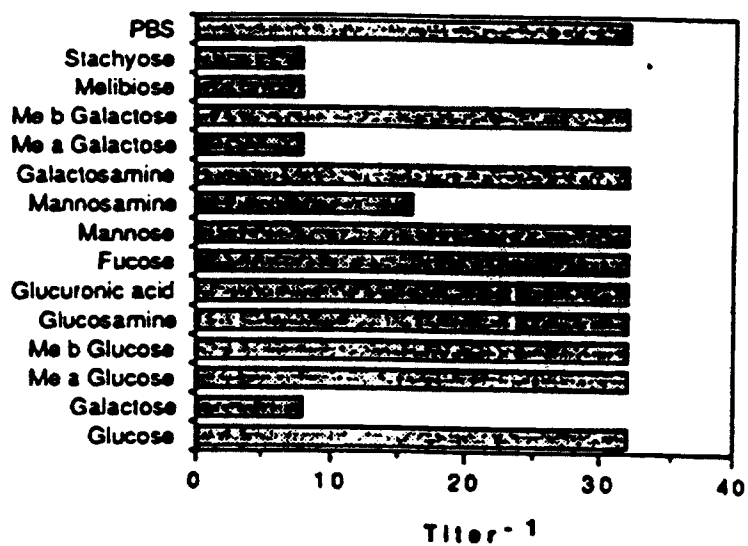


FIGURE 4



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FIGURE 5

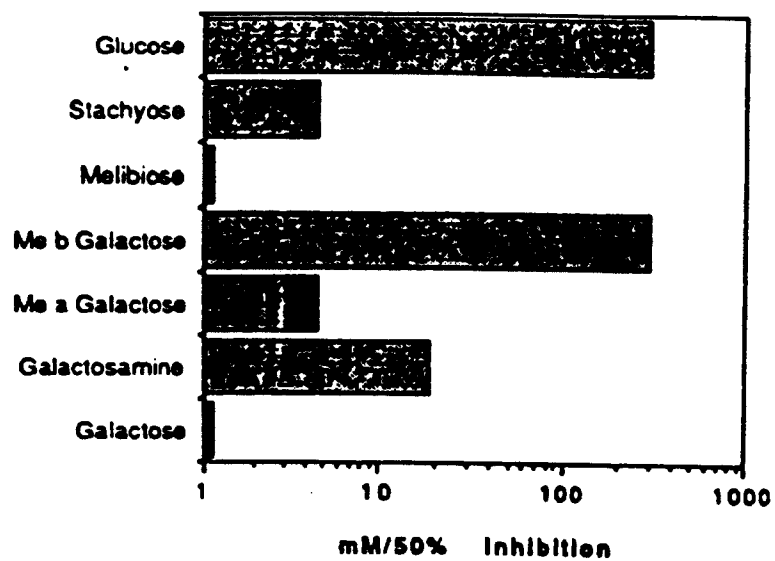
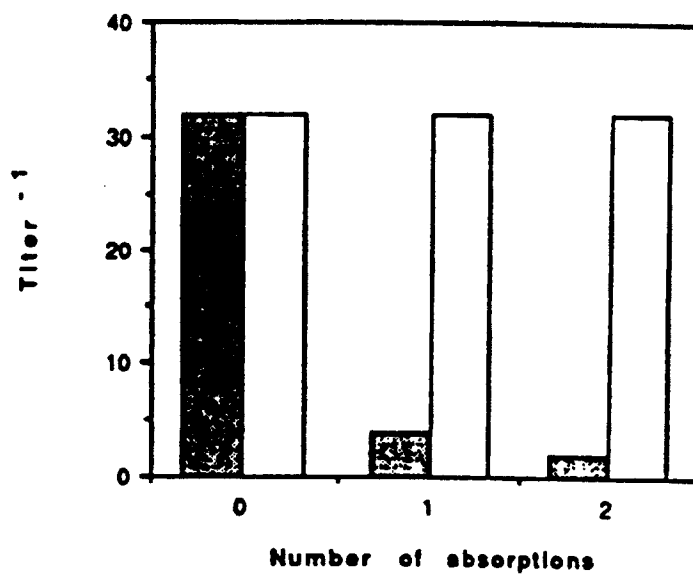


FIGURE 6



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FIGURE 7

EcoR1
BamH1
Hind111
Pst1
Kpn1
BstE11

8.5 —
6.1 —
4.8 —
3.6 —
2.8 —
2.0 —
1.5 —
1.2 —
1.0 —

